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13. ABSTRACT (Maximum 200 Words)

The human genome contains about 1% human endogenous retroviruses (HERV) sequences. Most HERVs are defective because of multiple termination codons. Of the many HERV families, only K appears to have all structural features necessary for viral replication. In the current project, HERV-K molecules that serve as novel tumor targets of human breast cancer will be evaluated. It is very critical to determine whether or not HERV-K protein is expressed in breast cancer. The expression of HERV-K env surface protein in human breast cancer tissues was evaluated by immunohistochemistry using anti-HERV-K antibodies. We detected protein expression in breast cancer tumor epithelial cells, but not in uninvolved epithelial cells, thus providing evidence that HERV-K env gene has translational activity in breast cancer. Furthermore, we detected anti-HERV-K env protein antibody in breast cancer patients, but not in normal female controls. This data provides indirect evidence of the expression of HERV-K env protein in human breast cancer. Also, the data demonstrate that the HERV-K env protein is immunoginic in breast cancer patients. These data support the hypothesis that HERV-K env protein may be a novel molecular target for detection, diagnosis, and immunotherapy of breast cancer.

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SUBJECT: Annual Report for Award Number DAMD17-00-1-0123
TITLE: HERV-K 10 as a target of immunotherapy for breast cancer
P.I. Feng Wang-Johanning, M.D.

INTRODUCTION:

1. Human endogenous retroviruses: Human endogenous retroviruses are descendants of exogenous retroviruses that became cellular genes by integration into host germ line cells (1). The human genome consists of about 1% retroviral sequences (2). Most of these human endogenous retroviruses (HERVs) are defective due to evolution (3); however, some HERV families contain open reading frames for all essential retroviral proteins whose purpose in tumor cells is unknown (4). Those HERVs that may be transcriptionally active in human include HERV-K, ERV3, HERV-E, HERV-H and HERV-W. Of the many HERV families, only K appears to have the full complement of open reading frames typical of replication competent mammalian retroviruses (4, 5). HERV-K has homology to the mouse mammary tumor virus (MMTV), which has been identified as an etiological agent of mammary cancer in inbred mice (6). HERV-K retrovirus-like particles have been described which encode for viral particles in the teratocarcinoma cell line GH (7, 8), and the breast cancer cell line T47D (9). The K family contains an additional ORF termed cORF (central), whose translated product is biologically comparable to the human immunodeficiency virus type-1 (HIV-1) Rev protein, critical for nuclear export of unspliced mRNA (8, 10-13). A causal relationship between endogenous retroviruses and human cancer has been explored, including the recent demonstration of the transforming ability of an HERV-K central open reading frame gene (*cORF*) (14). In mice, endogenous retroviral genes are transcriptionally silent in normal tissues but expressed in several well-studied murine tumor models (15, 16). In some of these models, the expressed env protein acts as a tumor antigen capable of inducing both antibody and T cell responses (17-20). In a mouse model system, tumor cells expressing a retroviral envelope protein escaped immune rejection and resulted in tumor growth *in vivo* (21). Their results emphasize the close relationship between endogenous and infectious retroviruses and might be important in relation to the process of tumor progression in humans (22).

HERV-K: HERV-K: The type K family (HERV-K) is present in an estimated 30 to 50 copies per human genome and includes some elements with long open reading frames (5), (23). This family of HERVs was originally identified by its homology to the mouse mammary tumor virus (MMTV), and contains members which are transcriptionally active in several human cancer tissues (2, 7, 8) as well as tumor cell lines, most notably the human breast cancer cell line T47D (24, 25). HERV-K genomic sequences were first detected in 1986, and HERV-K retrovirus-like particles have been described which encode for viral particles in the teratocarcinoma cell line GH (7, 8), and the breast cancer cell line T47D (9). HERV-K 10, a full-length proviral clone, has been sequenced and found to be defective (4). Several other reports suggest the existence of at least ten different sequence families in the human genome with varying degrees of homology to HERV-K10 and mouse mammary tumor virus (MMTV) *pol* genes, as well as to each other (26-28). It has been suggested that there are between 30 to 50 copies of HERV-K genomic DNA within the human genome; more recently this number has been increased to 170 (29). The HERV-K family of endogenous retroviruses is believed to be unique in several regards. Of the many HERV families, only K appears to have the full complement of open reading frames typical of replication competent mammalian retroviruses (LTR-gag-prt/pol-env-LTR) (4);(5).

   In teratocarcinoma cell lines, HERV-K *gag* genes are expressed, leading to the production of viral core proteins and virus particles (8). HERV-K is expressed in a complex splicing pattern in testicular tumors and derived cell lines as well as placenta. The HERV-K genome is also spliced into subgenomic transcripts in the human breast cancer cell line T47D (25). The K family contains an additional ORF termed cORF (central),

whose translated product is biologically comparable to the human immunodeficiency virus type-1 (HIV-1) Rev protein, critical for nuclear export of unspliced mRNA (8, 10-13).

In addition, an antibody response to the *gag* and *env* proteins of HERV-K has been identified in patients with seminoma, suggesting synthesis of these proteins *in vivo* (30), whereas healthy individuals and patients with inflammatory diseases very rarely develop such antibodies. The antibody titers of the patients showed a decrease with time after the patients received antitumor treatment or tumor removal (31). All these findings collectively indicate that HERV-K proteins constitute markers specific for germ cell tumors, but leave open the possibility that individual proteins exert functions that actively contribute to tumorigenesis.

BODY:

Hypothesis: Our hypothesis is that human endogenous retrovirus K (HERV-K) *env* protein might be an appropriate target for immunotherapy of breast cancer.

To test our hypothesis, we propose the following three Specific Aims:

Specific Aim 1. Detect or characterize HERV-K *env* protein expression in malignant, benign, or normal breast tissues:
Task1. To purify large amounts of HERV-K10 *env* gene recombinant proteins (months 1-6)
Task 2. To produce polyclonal and monoclonal antibodies against HERV-K10 *env* protein (months 7-12)
Task 3. To identify HERV-K10 *env* protein expression in breast tissues using anti-HERV-K10 antibodies (months 13-18)

Specific Aim 2. Analyze breast cancer patient sera (and appropriate controls) for antibody to HERVs

Task 1. To identify anti-HERV-K10 *env* antibody in patients with breast cancer or other disorders as well as normal female controls by ELISA and Western blot (months 19-24)
Task 2. To identify HERV-K10 *env* fusion proteins in a prokaryotic system using the antisera from breast cancer patients (months 19-24)

Specific Aim 3. Determine ability to induce human lymphocytes to become immune to HERV proteins using HERV-K 10 transfected autologous dendritic cells for *in vitro* sensitization:
Task 1. Generate the dendritic cells from peripheral blood mononuclear cells (PBMCs) (months 25-36)
Task 2. In vitro transcription of HERV-K 10 mRNA (months 25-36)
Task 3. Transfection of DCs with RNA (months 25-36)
Task 4. Induce and assess HERV-K10 antigen-specific T cell activity (months 27-36)

KEY RESEARCH ACCOMPLISHEMENTS:

a. Expression of both types of HERV-K env transcripts in human breast cancer tissues: So far, we demonstrated that multiple variants of both types HERV-K were transcriptionally activated in breast cancers. No clones with identical sequences could be detected in multiple samples from the same or different cancer tissue samples (Table 1).

Table 1. cDNA sequence analysis of a breast cancer sample (invasive ductal carcinoma)

| Transcripts	Primer used	Clone No.	Homology	HERV-K	Accession	nt
Env region	K-type 1	165K10C3	100%	K102	AF164610.1	6491-7552
(type 1)	K-type 1	165AC4E	99%	K102	AF164610.1	6491-7575

	K-type 1	165KC2	99%	K102	AF164610.1	6471-7575
Env region	K-type 2	165K22EC	98%	K109	AF164615.1	6660-7501
(type 2)	K-type 2	165K22C11	97%	K (I)	AB047209.1	7533-8680
	K-type 2	165K22C18	98%	K (I)	AB047209.1	7533-8680
	K-type 2	165K22C6	98%	K109	AF164615.1	6659-7194
Spliced	U5 &Env A	165UA2	99%	cORF	X82271.1	12-1505
subgenomic	U5 &Env A	165UA3	99%	K102	AF164610.1	833-879/5989-6658
Type 1 &	U5 &Env A	165UA4	99%	HML.2	AF164610.1	833-1076/6433-6949
Type 2	U5 &Env A	165UA5	99%	HML.2	AF164610.1	6688-6949
	U5 &Env B	165EBC22	99%	K102	AF164610.1	835-1075/8117-8186
Full-Length	P1/P3	165P1C2	98%	K102	AF164610.1	6072-7690
(Type 1)	P1/P3	165P1C8	95%	K (II)	AB047240.1	7016-8632

b. Multiple variants of HERV-K are transcriptionally activated in breast cancer samples: Three clones from Table 1 representing type 1 HERV-K were 99 to 100% homologous to HERV-K 102, with 1 to 3 bp differences (in a total 1,104 bp amplified). Meanwhile, of four clones representing type 2 HERV-K, two were 98% homologous to HERV-K 109 (28) with at least 13 bp differences in a total 1,194 bp, and two were 97 to 98% homologous to HERV-K (I) (32). These results suggest that multiple members of HERV-K from both types of HERV-K are transcriptionally activated in the same breast cancer patients, suggesting heterogeneity of HERV-K mRNA sequences in breast cancer. As a result of the large copy number of endogenous HERV-K *env* genes in the human genome, *env* gene heterogeneity may play a similar role in the pathogenesis of breast cancer. This sequence variation may reflect both mutation to escape the immune system and *in vivo* variation in biological properties.

c. Full-length and spliced env subgenomic sequences of HERV-K are transcriptionally activated in breast cancer samples: The last two clones in Table 1 represent full-length HERV-K transcripts detected using P1 and P3 primer pairs described previously (8) in breast cancer samples. They were 95 to 98% homologous to HERV-K (II) (32) and HERV-K 102 sequences. Full-length type 1 HERV-K expression was also verified by Northern blot analysis in our earlier study (see Paper #1). Full-length type 2 HERV-K expression was weaker than type 1, as assessed by RT-PCR using P1 and P3 primer, but could be detected by Northern blot using a probe specific to the 292 bp sequence characteristic of type 2 (data not shown). Expression of both types of full-length transcripts, with a 10-fold excess of full-length type 1 over type 2, had been demonstrated by others (25).

d. Analysis of putative subgenomic HERV-K transcripts: We found that both types of HERV-K genomes derived from breast cancer tissue are not defective in *env* splicing and are able to undergo splicing to subgenomic *env* transcripts (see Figure 1). This is in contrast to earlier reports which showed either only type 2 HERV-K splicing in teratocarcinoma cell lines, [i.e. that HERV-K type 1 geneomic sequences are defective in *env* splicing and may require the 292 bp sequence for splicing to occur (8)] or only type 1 HERV-K splicing in T47D breast cancer cell lines (25). These findings encouraged us to explore the HERV-K transcriptional profiles among breast cancer tissues examined.

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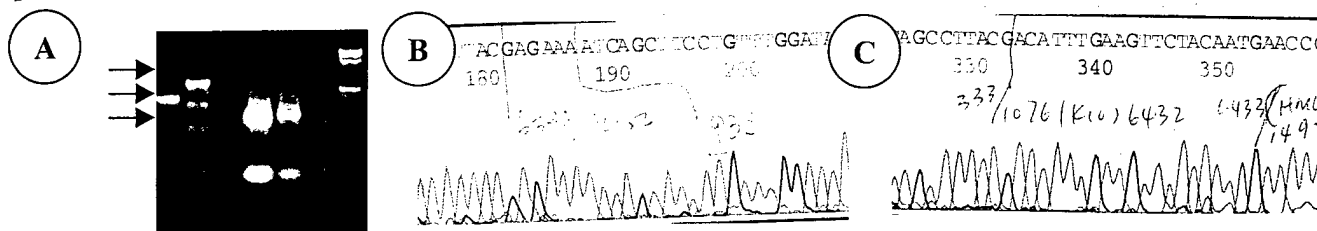
1, 2, 3, 4, 5, 6,M

Figure 1. Spliced HERV-K subgenomic *env* transcripts. A. RT-PCR products were obtained from breast cancer sample #1 (BC 1; lanes 1 and 4) and #2 (BC2; laness 2 and 5) by RT-PCR using U5 and *env* A (lanes 1 to 3) or B primers (lanes 4 to 6). Lanes 3 and 6 are negative controls. **B.** The SD (splice donor) and SA (splice acceptor) sites from breast cancer BC1 (lane 1) are located at bp numbers 927 and 6399. **C.** The SD and SA sites from breast cancer BC2 (lane 2) are located at bp numbers 1076 and 6432.

The PCR product (lane 1) in BC1 is approximately 497 bp and the PCR product in BC1 sample (lane 2) is approximately 790 bp. The difference between lane 1 and lane 2 is due to the 292 bp sequence present in BC2 RNA but not in BC1 sample. There is no difference between BC1 and BC2 samples in the 311 bp PCR products (lanes 4 and 5), as described previously (25, 33). This is the first report that both types of HERV-K RNA expression were detected in human breast cancer tissues, and that both types of HERV-K are able to undergo splicing to subgenomic transcripts.

e. Analysis of the splice donor and acceptor sites of HERV-K spliced transcripts in breast cancer cells and tissues: From Figure 1B and C, the SD and SA sites are different between type 1 and type 2 HERV-Ks. In lentiviruses and human T-cell lymphotropic virus-related viruses, small, multiply spliced mRNAs code for regulatory proteins involved in transcription, translation, and transport of viral RNAs. These findings prompted us to explore the question of the role of HERV-K in human breast cancer, and whether or not the spliced transcripts are capable of encoding proteins. A number of SD and SA sites were detected in different breast cancer cell lines and tissues (see Table 2). We also found a great degree of complexity in the *env* transcripts present, including the presence of unspliced transcripts, spliced transcripts with previously reported splice sites, and transcripts utilizing previously undescribed splice donor and acceptor sites. We are the first to show that both types of HERV-Ks are able to be spliced, and to identify a great variety of SD and SA in breast cancer samples.

Table 2. Analysis of splice donor and accepter in breast cancer cell lines and tissues

Tissue	165U2[1]	165U3[2]	165U4[1]	165U5[2]	177U26[1]	177U29[1]	178U11[2]	178U15[2]	165UB22	165UB23
SD	1076	878	1076	878	961	961	927	927	1076	1076
SA	6433	5997	6433	5997	6948	6948	6399	6399	8117	8117
ORF[6]	√	√	√	√	√	√	√	√	√	√

Cell lines	T47DU3[2]	T47U4[2]	MCF7c28[3]	231U3[3]	Tera 2U2[2]	Tera2U3[2]	Tera1U13[1]	Tera1U15[1]	Tera1UB4[1]
SD	883	883	2078	2078	1076	1076	1076	1035	1076
SA	6222	6222	7599	7599	6433	6433	6433	6756	6433
2nd SD[4]					6492	6492			
2nd SA[5]					6946	6794			
ORF[6]	√	√	N/A[7]	N/A	X	X	√	X	√

[1]Type 2 HERV-K, nt numbered according to HML-2 sequence (AF074086.2)
[2]Type 1 HERV-K, nt numbered according to HERV-K102 sequence (AF164610)
[3]Type 1 HERV-K, nt numbered according to HERV-K (II) sequence (AB047240)
[4]second splice donor
[5]second splice acceptor
[6]ORF: Open reading frame. √ means ORF is not defective, and X means ORF is defective.
[7]N/A could not be determined.

Table 2 identifies smaller *env* spliced subgenomic transcripts obtained from different breast cancer cells and tissues. These transcripts were sequenced and their SD and SA sites were detected. Most HERV-K env spliced subgenomic transcripts detected in our studies were type 1 (patients 165, 177 and 178 and breast cancer cell lines as described previously, Clinical Cancer Research, 2001). However, type 2 HERV-K *env* transcripts also are transcriptionally active (patient 165). Interestingly, we found that both types of HERV-K *env* mRNA were able to undergo splicing to subgenomic *env* transcripts. As already noted, this is in contrast to earlier reports which showed either predominately type 1 HERV-K splicing in T47D breast cancer cells (25), or only type 2 HERV-K splicing in teratocarcinoma cell lines (8). Four clones (165U2, 165U4, 165U3 165U5) derived from a breast cancer patient with invasive ductal carcinoma were sequenced. Clones #165U2 and 165 U4 belong to type 2, and 165U3 and 165U5 belong to type 1 HERV-K. This is the first report that both types of HERV-K are transcriptionally active in breast cancer tissues or tissues from other cancers. To date, all of the transcripts from clones derived from breast cancer patients and cell lines encode an open reading frame. In addition, no stop codon was found in these clones. Although some clones from the teratocarcinoma cell lines Tera 1 and Tera 2 had a second splice donor and splice acceptor site, we did not find the second splice sites in any clones derived from breast cancer cell lines and tissues. These results are in agreement with those reported by Etkind et al. (1). In teratocarcinoma cell lines, both types of HERV are transcribed, but only type 2 HERV-K splicing produces ORFs with no stop codon. Thus, only type 2 HERV transcripts in teratocarcinoma cells are non-defective, a finding which matches the results obtained by Lower et al. (2).

Recently, a novel gene, np9, resulting from a unique, HERV-K type 1-specific splice donor site, was confirmed by another research group to be expressed in various tumor tissues and transformed cell lines but not in normal, nontransformed cells (34). The highly specific expression of np9 with an HERV-K env-reading frame in tumor tissue suggests that the protein may function in tumorigenesis. Their results provide evidence that the numerous spliced variants of HERV that we have recently detected may also play important roles in breast tumorigenesis. It is possible that some of these variants will possess tumorigenic properties similar to the np9 variant just reported.

cORF is a protein encoded by the C-terminal open reading frame within the HERV-K env gene. This protein supports tumor growth in nude mice and associates with promyelocytic leukemia zinc finger protein (14). In Table 2, the sequences of clones 165U2, 165U4, Tera2U2, Tera2U3, Tera1U13, and Tera1UB4 are 99% homologous to central open reading frame (cORF) sequences. The translated product of cORF is biologically comparable to the human immunodeficiency virus type-1 (HIV-1) Rev protein, which is critical for nuclear export of unspliced mRNA (10-14). We have observed cORF mRNA expression in the breast cancer tissue samples of Table 2.

f. Production of HERV *env* protein in eukaryotic or prokaryotic vectors:
f-1. Expression of HERV *env* proteins in eukaryotic vectors: As a test of the coding potential of the above expressed *env* sequences, variant HERV *env* sequences were cloned into a eukaryotic expression vector for assay by coupled *in vitro* transcription-translation with [^{35}S] methionine. An HERV-K *env* protein of 40,000 daltons was observed in most clones of these HERV-K *env* cDNAs (see Figure 2A), and HERV-E *env* protein of 41,000 daltons was observed in some of these HERV-E *env* cDNAs (see Figure 2B).
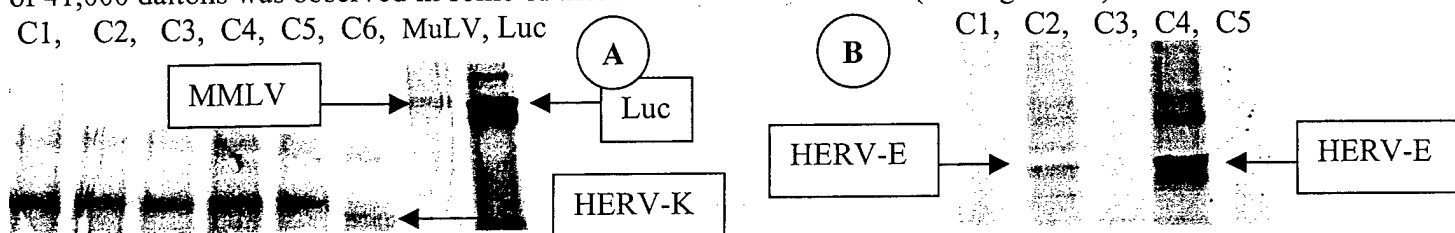
Figure 2. Production of HERV-K or HERV-E *env* protein from a eukaryotic vector. *In vitro* transcription-translation of HERV-K (**A**) or HERV-E (**B**) *env* proteins was carried out using the TNT T7 coupled reticulocyte system with [^{35}S] methionine (Promega). HERV-K or HERV-E *env* cDNAs in the pcDNA3 vector containing a Kozak sequence were used for the synthesis of HERV-K or HERV-E *env* protein. Two μl of reaction mixture were assayed by SDS-PAGE and autoradiography. **A.** Breast cancer clones C1 to C5 produced 40,500 dalton HERV-K *env* SU proteins, and C6 produced a 40,200 dalton protein. Lane MMLV is a murine endogenous *env* protein (72,000 daltons; gp70) and lane Luc is a luciferase protein (61,000 daltons) positive control. **B.** Prostate cancer clones from C2 to C4 produced 41,000 dalton HERV-E *env* SU proteins.

Whereas expression of various HERV mRNA species has been reported previously in different tumor tissues, HERV protein expression has been confirmed in only a minority of cases. Most HERV loci are thought to be defective, and certain HERV-K *env* sequences, including HERV-K10, HERV-K107, HERV-K (I), and HERV-K(II), are reported to contain at least a premature stop codon that ablates *env* protein expression. However, most *env* cDNAs derived from breast cancers were able to produce the expected size HERV-K *env* SU proteins in our *in vitro* system (Figure 2A). No stop codons were observed over the entire *env* region analyzed in the presumed reading frame, as confirmed by sequence analysis.

f-2. Expression of HERV *env* proteins in prokaryotic vectors: Various HERV *env* cDNAs derived from ovarian cancer tissues were cloned in the bacterial expression vector systems pQE (with 6His; Qiagen), and GST (Pharmacia). Some clones produced recombinant fusion proteins, such as HERV-K-His fusion surface protein (40,000 daltons; Figure 3A), GST-ERV3 *env* fusion protein (89,450 daltons = 63,450 for ERV3 *env* protein plus 26,000 daltons for GST; Figure 3B), and HERV-K *env* spliced protein (cORF-His; 14,000 daltons; Figure 3C).
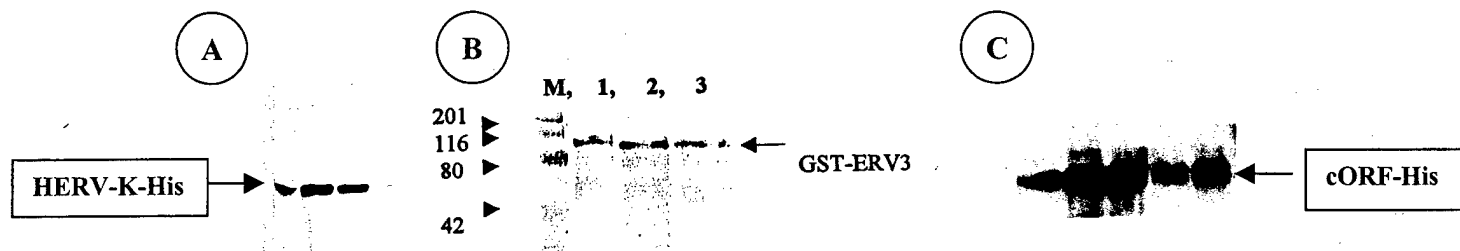

Figure 3. Western blot analysis of HERV fusion proteins. A. HERV-K SU-His Fusion protein (40 kDa). **B.** GST -ERV3 fusion protein (90 kDa). **C.** HERV-K spliced protein-His fusion protein (14kDa). Several HERV *env* proteins have been produced in our laboratory including ERV3, HERV-E, and HERV-K. HERV-K *gag* and smaller spliced HERV-K env proteins have also been produced in our laboratory. The authenticity of HERV *env* fusion proteins was further confirmed by sequence analysis.

Several HERV *env* proteins have been produced in our laboratory including ERV3, HERV-E, and HERV-K. HERV-K *gag* and smaller spliced HERV-K env proteins have also been produced in our laboratory. The authenticity of HERV *env* fusion proteins was further confirmed by sequence analysis.

g. Production of anti-HERV antibodies by immunizing animals with purified HERV fusion proteins: These proteins were purified according to the manufacturer's directions, and used to immunize New Zealand White rabbits to produce polyclonal antisera, and to immunize Balb/c mice to produce monoclonal antibodies. If the HERV-K-His fusion protein was used to immunize the animals, the same relative amount of HERV-K GST fusion protein was used to screen for anti-HERV-K polyclonal (pAb) and monoclonal antibody (mAb)

production. After anti-HERV sera with the highest specificity and sensitivity were chosen, they were further purified with protein G immobilized by the CNBr method to Sepharose 4 fast flow (Pharmacia Biotech). Several anti-HERV polyclonal antibodies have been produced so far, including anti-HERV-K *env* pAb, anti-HERV-E *env*, anti-HERV-K *gag* pAb, anti-ERV3 pAb, and anti-HERV-K cORF pAb. However, these polyclonal anti-HERV antibodies obtained from rabbits have a cross reaction with other HERV fusion proteins. Monoclonal antibodies against HERV were also produced in our laboratory, including anti-HERV-E *env*, anti-HERV-K *env*, and anti-HERV-K *gag* mAbs. The antiserum from a mouse immunized with 6His-HERV-K fusion protein was used to test for specificity against HERV-K *env* protein by ELISA analysis (see Figure 4).
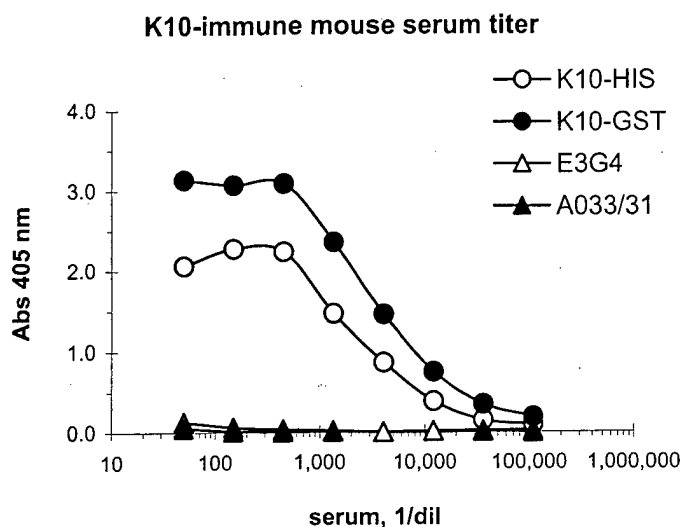

K10-immune mouse serum titer

—O— K10-HIS
—●— K10-GST
—△— E3G4
—▲— A033/31

Figure 4. Binding affinity or specificities of anti-HERV-K sera: ELISA analysis of binding affinity or specificities of the antisera obtained from a mouse immunized with HERV-K *env* -6 His fusion protein. The ELISA plate was coated with various HERV fusion proteins (10 μg per ml, 100 μl per well) including K10G17Q18 (K10-His, HERV-K *env* SU cDNA in a 6-His vector), K10G17 (K10-GST, HERV-K *env* SU cDNA in a GST vector), ERV3G4 (E3G4, ERV3 *env* SU cDNA in a GST vector), and HERV-E4-1Q4 (A033/31, HERV-E4-1 *env* SU cDNA in a 6-His vector). Antisera were diluted from 50 to 109,350. The ELSIA plate was read at a wavelength of 405 nm.

The sera reacted only with 6His-HERV-K fusion protein (K-HIS, in pQE30 vector) and GST-HERV-K protein (K-GST, in GST vector), but not with GST-ERV3 (E3-GST, ERV3 *env* protein in a GST vector) or 6His-HERV-E fusion protein (E-His, HERV-E *env* protein in a pQE vector).

The spleen cells obtained from this mouse have been fused and used to produce anti-HERV-K hybridoma. ELISA using various HERV env fusion proteins was used to test the clones for anti HERV-K specificity and sensitivity against HERV-K proteins. Several clones with high sensitivity and specificity toward HERV-K env protein were further cultured and expanded (see Figure 5). These clones were tested by ELISA, and those clones that produced anti-HERV-K antibody were retained and will be used to grow and produce monoclonal antibody. Monoclonal antibody will be further purified and used to detect the expression of HERV-K proteins.

Figure 5. Specificity of anti-HERV-K monoclonal antibody: Anti-HERV-K monoclonal antibodies produced in our laboratory were tested for specificity and sensitivity against HERV-K env 6His fusion protein and compared with other 6His HERV env fusion proteins by ELISA.

Anti-HERV-K mAb Specificity

□ K-His
▣ E-His
□ 3-His

HERV-K hybridoma Clones

10
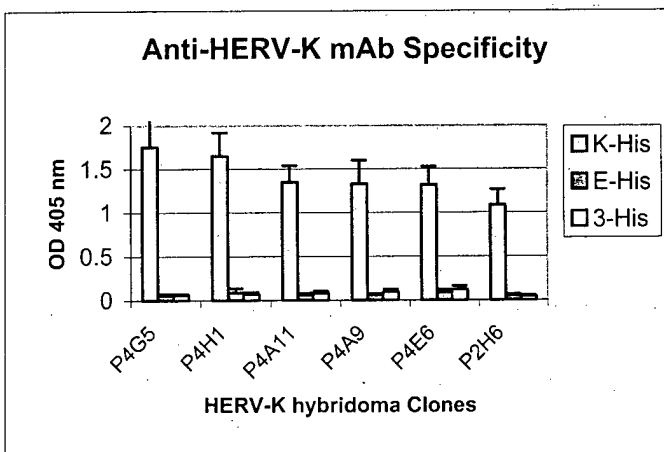
h. Detection of HERV-K *env* protein expression in human breast cancer tissues: This entire grant proposal is based on the hypothesis that HERV-K *env* mRNA is not only actively transcribed in human breast cancer tissues, but is also translated to protein in these tissues. It is thus very critical to test for HERV-K protein expression in human breast cancer tissues. To date, expression of HERV-K *env* protein in breast cancer has not been reported. In this proposal, anti-HERV-K polyclonal antibodies were used successfully to detect HERV-K *env* protein expression in human breast cancer tissues. Characterization of HERV-K *env* translational activity was employed in serial breast tissue sections by immunohistochemistry using our polyclonal antibody. HERV-K *env* protein was detected only in tumor epithelial cells derived from ductal carcinoma *in situ* or invasive ductal carcinoma tissues, but not in adjacent uninvolved epithelial cells from the same patient tissue sample (see Figure 6).
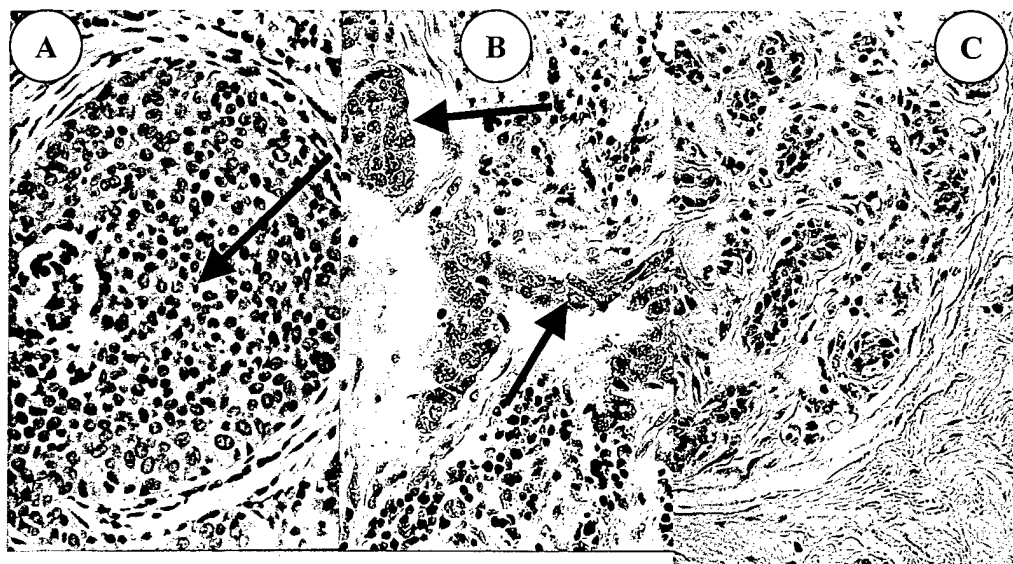
Figure 6. Detection of HERV-K env protein expression in breast biopsies by immunohistochemistry. Serial breast tissue sections obtained from a breast cancer patient were assessed by immunohistochemstry using a polyclonal antibody specific against HERV-K env protein. **A.** Ductal carcinoma in situ (DCIS): **B.** invasive ductal carcinoma (IDC): **C.** Uninvolved breast tissues. The expression of HERV-K env protein was detected only in tumor epithelial cells including DCIS (A) and IDC (B), but not in uninvolved normal epithelail cells (C). This result provided further evidence that the expression of HERV-K protein is tumor-specific.

In addition, we did not detect the expression of HERV-K *env* protein in some of the normal breast tissues we tested. These results matched the results we observed by RNA *in situ* hybridization using HERV-K antisense probe (See Figure 7)

Figure 7. Detection of HERV-K transcripts in breast specimens by *in situ* hybridization. Characterization of HERV-K transcriptional activity in serial b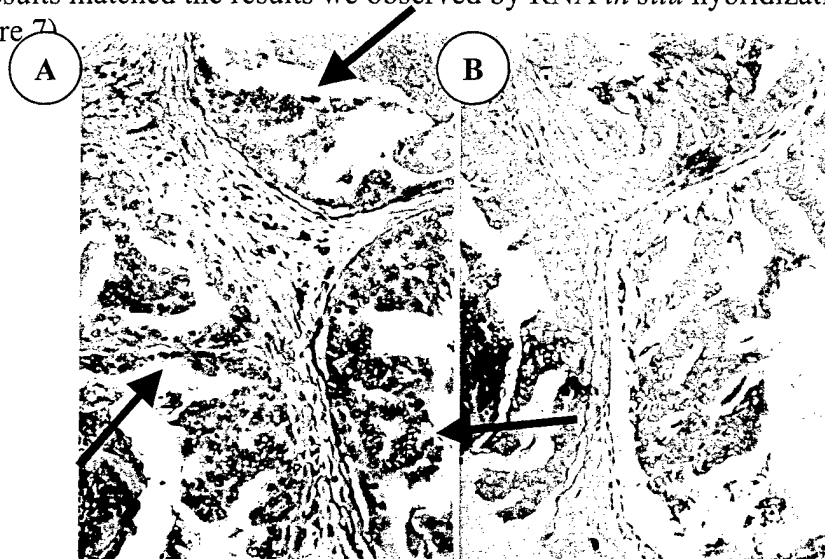reast tissue sections by *in situ* hybridization using a digoxigenin-labeled HERV-K *env* RNA probe. **A** and **B.** breast ductal carcinoma *in situ*. Section depicted in A was hybridized with HERV-K *env* RNA antisense probes (Figure 7A), while that depicted in B was hybridized with HERV-K RNA sense probes (Figure 7B). Sections were visualized by light microscopy.

These results demonstrate that the HERV-K *env* gene is expressed in neoplastic breast tissue not only at the level of mRNA, but also at the protein level. This conclusion was further confirmed in next section.

i. Detection the Anti-HERV-K antisera in patients with breast cancers: In order to demonstrate that HERV-K proteins are immunoginic in their host, the sera obtained from various cancer patients were used to test for anti-HERV-K or other HERV proteins by ELISA and Western blot.
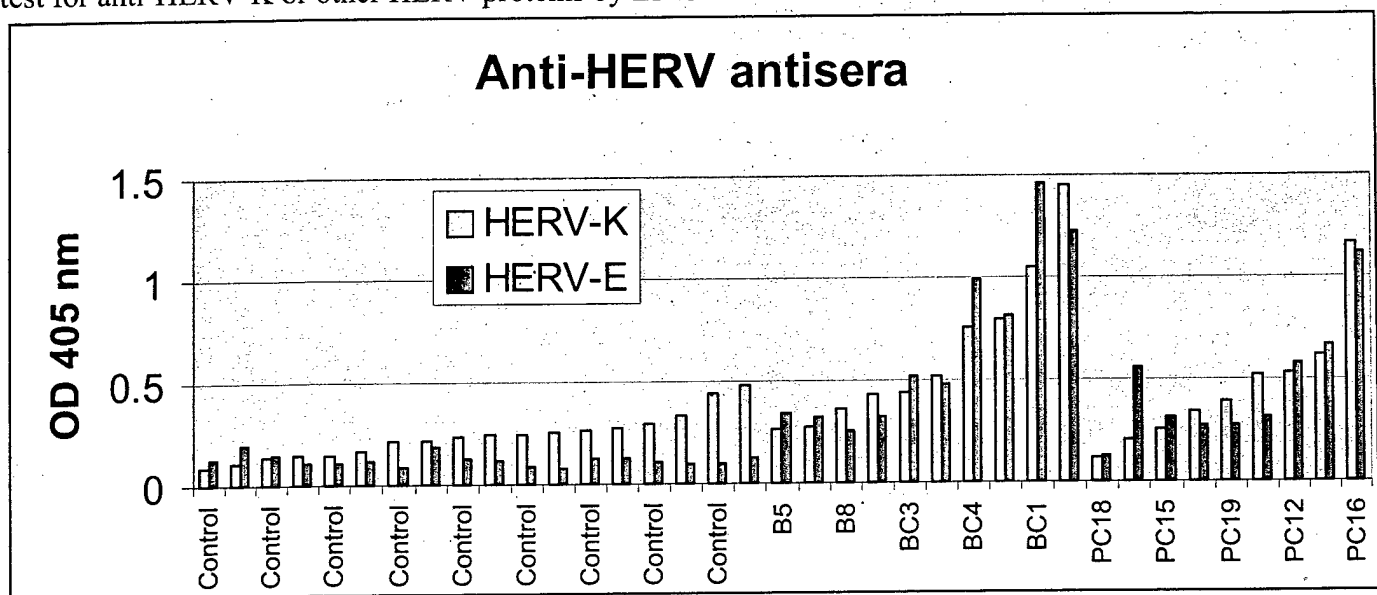
Figure 8. Binding affinity and specificity of anti-HERV-K or anti-HERV-E env protein sera: ELISA analysis of binding affinity and specificity of the sera obtained from normal controls (control), breast cancer patients (BC1 to BC10) and prostate cancer patients (PC11 to 19). The ELISA plate was coated with HERV *env* fusion proteins including HERV-K and HERV-E (10 µg per ml, 100 µl per well). Patient sera were diluted 1:200. The ELSIA plate was read at a wavelength of 405 nm.

Our ELISA result from Figure 8 showed that no anti-HERV-K or anti-HERV-E OD value was higher than 0.5 in 18 normal control sera, but anti-HERV-K or anti-HERV-E OD values higher than 0.5 were observed in 4 of 10 total breast cancer patient sera (40% positive) and anti-HERV-K or anti-HERV-E OD values higher than 0.5 were seen in at least in one prostate cancer patient out of a total of nine patients. Our results clearly show that anti-HERV-K or anti-HERV-E env protein antibodies are present in breast cancer patients. These data provide indirect evidence of the possibility of expression of HERV-K env proteins in breast cancer patients. Also, the data indicate that HERV-K env proteins are immunogenic in breast cancer patients. So far, a total of about 150 sera were used to screen for anti-HERV env protein antibodies. Anti-HERV-K env antibodies were detected

only in breast (30-40 % N = 50), ovarian (30-40% N = 30), and prostate cancer patients (10-15% N = 20), but not in normal controls (N = 50). We plan to screen more sera samples to confirm our results.

j. Detection of expression of HERV env mRNA and protein in stably transfected cells by Northern blot and immunoprecipitation: Cells were stably transfected with various HERV *env* genes cloned into a mammalian CMV promoter expression vector (pSecTagB or pSecTagC for in-frame expression; Clontech Inc.). Various HERV *env* genes were transfected, including ERV3 (1,793 bp), HERV-E (1,071 bp), HERV-K plus (386 bp), and HERV-K surface protein (1,058 bp). Stable transfectants were selected using Zeocin as described previously (see Appendix paper 2; Cancer Res., 1998). The expression of HERV *env* mRNA or HERV *env* proteins in selected cells were detected by RT-PCR and Northern blot (Figure 9) using specific HERV *env* primers and probes or by immunohistochemistry (IHC) or immunoprecipitation (Figure 10) using anti-HERV antibodies.
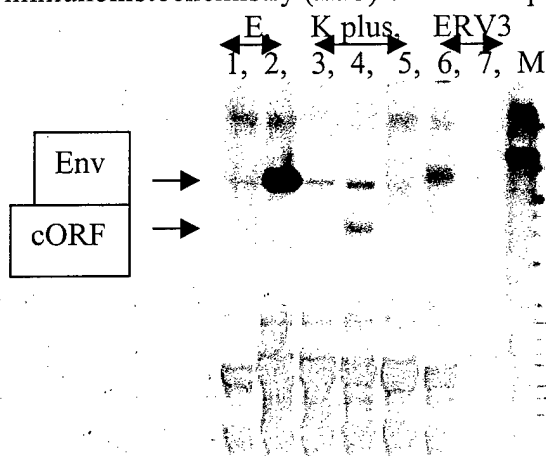

Figure 9. Northern blot analysis of HERV *env* mRNA expression in cells stably transfected with various HERV *env* genes cloned into mammalian CMV vectors. The expression of various HERV *env* RNA transcripts was detected by Northern blot using multiple *env* probes (HERV-E, HERV-Kplus, and ERV3 *env* genes) labeled with ^{32}P. The orders of lanes are HeLa-pSecTagB (lane 1), HeLa-HERV-E-pSecTagB (lane 2), OV4-HERV-Kplus-pSecTagB (lane 3), MCF-7-HERV-Kplus-pSecTagB (lane 4), Rat-1-K-plus-pSecTagB (lane 5), HeLa-ERV3-pSecTagC (lane 6), Rat-1-pSecTagB (lane 7). M is the RNA marker (far right lane; Millennium Marker; Ambion Inc.). The HERV-E *env* RNA band in HeLa-HERV-EpSecTagB is 3.4 Kb, (see lane 2). The HERV-K *env* RNA bands in MCF-7-HERV-Kplus-pSecTagB are 3.1 Kb (for top band) and 1.6 Kb (for bottom band; see lane 4). The ERV3 *env* RNA band is 3.8 Kb (lane 6). To verify the presence of RNA in all lanes, the blot was stained with methylene blue solution (lower panel).

The Northern blot data demonstrate that HERV *env* RNA was expressed in cells stably transfected with various HERV *env* genes. In transfected MCF-7 cells, both transcripts (env and central open reading frame) were detected due to the presence of a spliced *env* gene.

The presence of HERV env proteins in the supernatant of these stably-transfected cells was examined by ELISA (data not shown), and translated protein in the cell lysates was detected by immunoprecipitation as described previously (see Appendix paper #3) using anti-HERV specific antibodies (see Figure 10). These data provide strong evidence that cells transfected with *env* genes are capable of expressing the HERV mRNA, and of translating the mRNA to proteins.
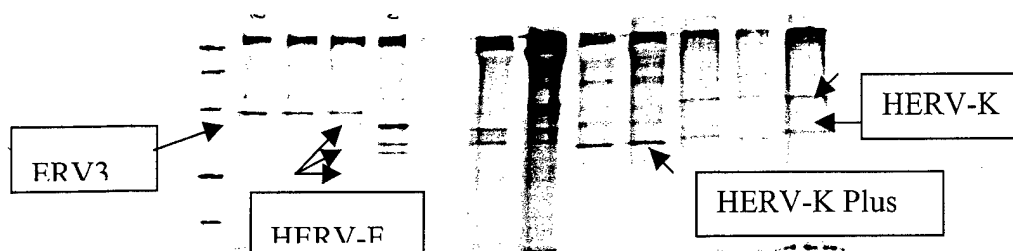
Figure 10. Detection the expression of HERV-env proteins in cells stably transfected with various HERV env genes cloned into pSecTag vectors: Immunoprecipitation analysis was employed to detect the expression of HERV env proteins using anti-HERV polyclonal antibodies. ERV 3 env protein (73 KDa) was detected in Rat-1 (lane 1), MCF-7 (breast cancer cells, lane 2), and HeLa (cervical cancer cells, lane 3) stably transfected with ERV3 *env* gene, using anti-ERV3 polyclonal antibody (Rabbit # 2442). HERV-E env protein (M_r of the three bands: 63, 52, and 48 KDa) was detected in MCF-7 (lane 4) and OV4 (lane 5: bands can be seen after longer exposure) cells stably transfected with HERV-E *env* gene, using anti-HERV-E polyclonal antibody (Rabbit # 5654). HERV-K (77, 40, 31KDa) env proteins were detected in Rat-1 (lane 6,7, 10 and 12) cells stably transfected with HERV-K *env* genes derived from different clones, using anti-HERV-K polyclonal antibody (Rabbit #5691). HERV-K plus (45 KDa) env proteins were detected in Rat-1 (lane 8) and MCF-7 (lane 9) cells stably transfected HERV-K plus, using anti-HERV-K polyclonal antibody (Rabbit #5693).

In this section, we further demonstrated that we could express various HERV env genes in mammalian cell lines, and that our anti-HERV env protein antibodies could detect the expression of various HERV env proteins in our system. To date, no other research group has available the various HERV env proteins and anti-HERV env antibodies that we have generated. These HERV env proteins and antibodies will provide us with a unique opportunity to carry out research defining the role of the HERV-K env gene in human breast cancer.

REPORTABLE OUTCOME:

None.

CONCLUSION:

In summary, we successful produced polyclonal antibody specific against HERV-K *env* surface protein and some monoclonal antibodies against this protein. It is very important that our data so far provide evidence that HERV-K *env* is not only transcriptionally active in breast cancer, but also has translational activity in breast cancer. Furthermore, our data from breast cancer patients provide more evidence for the expression of HERV-K env protein in breast cancer patients, but not in normal controls. Very importantly, HERV-K env protein is immunogenic in breast cancer patients. Our results support the hypothesis that HERV-K env protein may be a novel molecular target for detection, diagnosis, and therapy of human breast cancer.

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Advances in Brief

Expression of Human Endogenous Retrovirus K Envelope Transcripts in Human Breast Cancer[1]

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Abstract

Purpose: We investigated the expression of human endogenous retroviral (HERV) sequences in breast cancer.

Experimental Design: Reverse transcription-PCR (RT-PCR) was used to examine expression of the envelope (*env*) region of ERV3, HERV-E4-1, and HERV-K in breast cancer cell lines, human breast tumor samples, adjacent uninvolved breast tissues, nonmalignant breast tissues, and placenta. Expression of HERV transcripts was confirmed by Northern blot analysis and *in situ* hybridization (ISH). To evaluate coding potential, amplified HERV sequences were cloned into vectors for expression and sequence analysis.

Results: No expression of ERV3 or HERV-E4-1 RNA was detected in the analyzed breast samples. In contrast, HERV-K transcripts were detected in most breast cancer cell lines and many breast tumor tissues. Expression was detected in a small percentage of matched, uninvolved breast tissues and in placentas but not nonmalignant breast tissues. In HERV-K-positive breast cancer tissues, Northern blot analysis demonstrated full-length proviral and spliced *env* transcripts. ISH demonstrated expression of HERV-K transcripts in breast tumor cells but not in normal or uninvolved breast epithelial cells. Independently isolated clones of HERV-K *env* cDNA generated recombinant proteins of the expected size. Sequence analysis of *env* cDNA clones derived from four breast tumor samples revealed >97% identity with the type I HERV-K102, with no premature termination codons. Independent isolates from the same breast tumor sample showed nucleotide sequence differences, suggesting that multiple loci may be transcribed.

Conclusions: These data indicate that HERV-K transcripts with coding potential for the envelope region are expressed frequently in human breast cancer.

Introduction

HERVs[3] are stably inherited sequences thought to have entered the germ line of their host more than a million years ago (1, 2). These elements are widely dispersed throughout the genome and are estimated to comprise >1% of the entire human genome. Most HERVs are defective because of multiple termination codons and deletions (3), but some appear to contain all structural features necessary for viral replication (4). HERVs are grouped into single- and multiple-copy families, usually classified according to the tRNA used for reverse transcription (2). The type K family (HERV-K) is present in an estimated 30–50 copies/human genome and includes some elements with long open reading frames (5, 6). This family of HERVs was originally identified by its homology to the mouse mammary tumor virus and contains members that are transcriptionally active in several human cancer tissues (1, 7, 8) as well as tumor cell lines, notably in the human breast cancer cell line T47D (9, 10). Two general types of HERV-K genomes exist, distinguished by the absence (type 1) or the presence (type 2) of 292 nucleotides at the boundary of the putative *pol* and *env* genes (4, 10).

Additional endogenous retroviral sequences that may be transcriptionally active in humans include ERV3 and HERV-E. ERV3 is a single-copy, full-length provirus that contains a nondefective *env* glycoprotein gene (11) and functional long terminal repeats. ERV3 *env* mRNAs are expressed in placenta and some tumor cell lines (12–15). HERV-E is a multiple-copy HERV family that contains long open reading frames in the *pol* and *env* regions of the provirus, indicative of the potential for expression (16). Expression of transmembrane env protein from the prototypic HERV-E, HERV-E4-1, has been reported in some cancer cell lines including colon carcinoma, germ cell tumors, and prostate adenocarcinoma (17).

The widespread distribution of multiple endogenous retroviral elements in mammalian genomes suggests that they may perform significant biological roles in the host and thus have been evolutionarily conserved. Several functions for HERVs have been proposed. Reverse transcription and integration of retroviral elements may contribute to the plasticity of the genome, accelerating the evolution of new genes (18) and altering the transcription of existing genes (19). HERV-encoded proteins

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[3] The abbreviations use are: HERV, human endogenous retrovirus; RT-PCR, reverse transcription-PCR; env, envelope; HME, human mammary epithelial; ISH, *in situ* hybridization; GST, glutathione *S*-transferase; IDC, invasive ductal carcinoma.
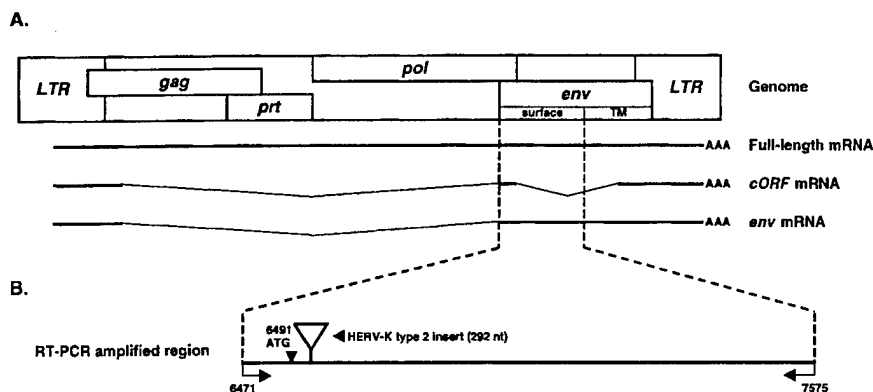
A.

B.

Fig. 1 HERV-K genome structure and RNA transcripts targeted by RT-PCR. *A*, generalized HERV-K genomic organization, showing only RNA transcripts that include *env* coding regions. *B*, diagrammatic representation of HERV-K *env* RT-PCR product targeted in the current study. Nucleotide numbering is according to the GenBank sequence accession no. AF164610.1 for HERV-K102 (35), a type I HERV. Note that only the putative surface env protein coding region was amplified. *TM*, putative transmembrane region of env.

may also have functions *in vivo*. In the placenta, the env protein of HERV-W has been shown to mediate the formation of syncytiotrophoblasts, an important step in placental morphogenesis (20). Expressed HERV proteins may also contribute to autoimmune disorders (21), although this remains controversial (22). A causal relationship between endogenous retroviruses and human cancer has been explored, including the recent demonstration of the transforming ability of an HERV-K central open reading frame gene (*cORF*; Ref. 23). In mice, endogenous retroviral genes are transcriptionally silent in normal tissues but expressed in several well-studied murine tumor models (24, 25). In some of these models, the expressed env protein acts as a tumor antigen capable of inducing both antibody and T-cell responses (26–29). In humans, tumors of germ cell origin have been reported to express HERV-K transcripts (30–32), and in seminoma patients, the HERV-K10 env protein is reported to be a tumor antigen eliciting host antibody responses (33).

As part of an ongoing search for tumor antigens important in breast cancer, we analyzed expression of the *env* region of ERV3, HERV-E4-1, and HERV-K in human breast cancer cell lines and tissues. Here we demonstrate that HERV-K transcripts are specifically and frequently expressed in human breast cancer, and that some of these transcripts contain open reading frames capable of producing env protein.

Materials and Methods

Cells and Tissues. Human breast cancer cell lines (BT-20, ZR-75-1, MCF-7, SKBr-3, MDA-MB-231, MDA-MB-453, BT-474, and T47D) were obtained from the American Type Culture Collection (Rockville, MD). Normal HME cells were obtained from Clonetics (San Diego, CA). Breast cell lines were cultured in the medium recommended by the American Type Culture Collection. The human teratocarcinoma cell lines Tera I and Tera II were kindly provided by Drs. Gail H. Vance and Virginia C. Thurston (Indiana University School of Medicine) and were cultured in α-MEM (Mediatech, Herndon, VA) with 10% fetal bovine serum (HyClone) and insulin (6 ng/ml; Sigma Chemical Co., St. Louis, MO). T47D cells were treated with 10 nM β-estradiol (Sigma Chemical Co.) and 100 nM progesterone (Sigma Chemical Co.) as described previously (34). Human breast tumor tissue, nonmalignant breast tissue from patients with other breast disorders (primarily fibroadenomas), normal

human breast tissue from reduction mammoplasty, and placenta were provided by the Tissue Procurement Shared Facility of the Comprehensive Cancer Center at the University of Alabama at Birmingham, with Institution Review Board approval. Representative samples from breast tumor tissue and adjacent uninvolved tissue were isolated based on gross inspection. Tissue samples were snap-frozen and stored at −70°C until RNA isolation. For *in situ* hybridization, both snap-frozen and formalin-fixed, paraffin-embedded tissues were used. For Northern blot analysis, RNA from normal breast tissue (pooled from eight individuals) was purchased from Clontech Lab, Inc. (Palo Alto, CA), and RNA from breast cancer tissues (invasive ductal carcinoma) was purchased from BioChain Institute, Inc. (Hayward, CA).

RNA Preparation. Total RNA from cell lines and breast tissue samples was isolated using RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX) following the protocol provided by the manufacturer. RNA was treated with DNase (RNase-free; 1–2 units of DNase I/μg of DNA; Ambion, Austin, TX) at 37°C for 30 min to remove contaminating DNA, followed by heating at 75°C for 5 min to destroy residual DNase activity.

PCR Primers. Oligonucleotide primers derived from the *env* sequences encoding the putative env surface protein of ERV3 (nucleotides 786-2530; Ref. 11), HERV-E4-1 (nucleotides 6211-7559; Ref. 16), and HERV-K (nucleotides 6471-7575; Ref. 4) were used to amplify cDNA prepared from human tissues and cell lines (Fig. 1). Primer sequences were as follows: ERV3 sense, 5′-ACACTACGTGTCGGGGAACATCATG; ERV3 antisense, 5′-ACCAACCTCTGAAAAGGGAATCTGG; HERV-E4-1 sense, 5′-CTGGTCCACGCACGGCCGAAGCA-TG; HERV-E4-1 antisense, 5′-AAAAGGACGACTTAATA-GAGCCAAT; HERV-K sense, 5′-AGAAAAGGGCCTCCA CGGAGATG; HERV-K antisense, 5′-ACTGCAATTAAAG-TAAAAATGAA. The HERV-K primers are 100% homologous to the following published HERV-K type 1 *env* sequences: K10 (GenBank accession no. M14123.1), K101 (GenBank accession no. AF16409.1), K102 (GenBank accession no. AF164610.1), K103 (GenBank accession no. AF164611.1), chromosome 5 (GenBank accession no. AC016577.4), and chromosome 19 (GenBank accession no. AC008996.5). These primers are expected to amplify both unspliced and spliced *env* transcripts. All HERV oligonucleotides were synthesized by Life Technologies,

Inc. (Grand Island, NY). Control primers to amplify human β-actin were from Stratagene (La Jolla, CA).

RT-PCR. Isolated RNA was incubated at 65°C for 10 min, followed by incubation on ice for 2 min prior to reverse transcription. Total RNA from each sample was reverse transcribed using cDNA synthesis beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) as per the manufacturer's directions. The reverse transcribed samples were amplified in a volume of 50 μl containing 3.3 μl of the first-strand cDNA synthesis mixture (corresponding to 1 μg of input RNA), 5 μl of 10× PCR buffer (Qiagen, Valencia, CA), 0.5 μl of Ampli-Taq DNA polymerase (2.5 units; Qiagen), and various sense and antisense oligonucleotide primer pairs at 50 pmol each. Each sample was analyzed in parallel with human β-actin primers. To assure that observations were not attributable to DNA contamination, all RNA samples were treated with DNase before cDNA synthesis. Additionally, 1 μg of RNA from each sample without reverse transcription was PCR amplified to control for genomic DNA contamination. PCR reactions were initially denatured at 94°C for 4 min, followed by 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1 min). Amplified products were analyzed on a 1% agarose gel.

Northern Blot Analysis. Samples of total RNA (15 μg/lane) were electrophoresed at 5 V/cm on a 1.2% agarose-formaldehyde gel. After electrophoresis, gels were washed and transferred overnight onto a positively charged nylon membrane (Roche Molecular Biochemicals, Indianapolis, IN) using a Transblotter apparatus (Schleicher and Schuell, Keene, NH) and 10× SSPE buffer (1.5 M NaCl, 0.1 M NaH$_2$PO$_4$, and 20 mM EDTA, pH 7.4). Membranes were stained with methylene blue solution to visualize 18 S and 28 S bands.

The probe for Northern blot analysis was prepared using an *env* cDNA derived from patient 1 breast tumor RNA (Fig. 6, *Pt1, clone #2*), corresponding to the HERV-K102 sequence from nucleotides 6471–7575 (GenBank accession no. AF164610.1). The cDNA was labeled with [^{32}P]dATP using the random-primed labeling method. Labeled probe was hybridized overnight at 62°C with the membrane, using a high-efficiency hybridization buffer (Molecular Research Center, Cincinnati, OH). Membranes were washed three times at room temperature with prehybridization/wash solution (1× SSC, 1% SDS), followed by three washes at 65°C with the same solution, and exposure to autoradiography film at −70°C for 24 h.

In Situ **Hybridization.** RNA probes were prepared from patient 1, HERV-K *env* clone #2 (Fig. 6). One μg of the linearized plasmid was used as template. The *in vitro* transcription and labeling of probe was performed at 37°C for 2 h with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals) using T7 and SP6 RNA polymerases to obtain run-off transcripts of the antisense (complementary to the mRNA) or sense (negative control) probes. Paraffin-embedded breast tissue specimens were cut into serial 5-μm sections, melted, deparaffinized in xylene, rehydrated in ethanol, and then fixed in 4% paraformaldehyde. Snap-frozen breast specimens were cut into 5-μm serial sections and fixed in 4% paraformaldehyde directly. After fixation, tissue sections were treated with proteinase K (20 μg/ml in 50 mM Tris-HCl, 5 mM EDTA) at 37°C for 15 min,

washed with PBS, and incubated at room temperature in 0.1 M triethanolamine-HCl plus 0.25% acetic anhydride. One section from each group was pretreated with RNase A (Sigma Chemical Co.) before proteinase K treatment as a control. Hybridization of RNase-treated sections with antisense RNA probe verified that RNA and not genomic DNA was the target of the hybridization. Tissue sections were equilibrated in Quick-Hyb hybridization buffer (Stratagene, La Jolla, CA) for 30 min. Antisense or sense riboprobes were denatured and added with salmon sperm DNA (250 μg/ml; Stratagene) to the tissue sections and incubated at 68°C overnight. Sections were washed twice with 0.2× SSC, 0.1% SDS and then once with STE buffer (0.5 M NaCl, 1 μM EDTA, and 0.02 M Tris) at room temperature. After treating with RNase A (50 μg/ml in STE buffer) at 37°C for 45 min to minimize nonspecific binding, the sections were washed once with STE and once in 0.1× SSC plus 0.1% SDS at 68°C for 15 min. Detection of digoxigenin-labeled nucleic acids was by enzyme immunoassay with a digoxigenin nucleic acid detection kit (Roche Molecular Biochemicals), using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad, Hercules, CA), followed by development at room temperature for 1–2 h in the dark. Sections were then rinsed in deionized water and counterstained with methyl green (Zymed, South San Francisco, CA) for 15 min. Adjacent sections were hybridized with sense probe as a negative control or with H&E for histological evaluation.

Synthesis of HERV-K env Fusion Protein. For construction of GST-fusion protein expression constructs containing partial HERV-K *env* sequences, the following modified RT-PCR primers were used: sense 5'-CCG<u>GAATTC</u>GTAA-CACCAGTCACATGGATG (nucleotides 6494), antisense 5'-ATAGTTTA<u>GCGGCCGC</u>TCTTTTGGATCTATTTAAAAC-ACC (amplifies nucleotides 6494–7552; GenBank accession no. M14123.1), with engineered recognition sequences for restriction enzymes (*Eco*RI and *Not*I, respectively) underlined. RT-PCR products from patient breast tumor samples were cloned into the pGEX 4T-1 GST gene fusion vector (Pharmacia). Plasmids with predicted *env* insert size were screened for protein production on a small scale using isopropyl-β-D-thiogalactopyranoside induction. GST fusion proteins were purified by affinity chromatography using glutathione Sepharose 4B (Pharmacia), subjected to 12% SDS-PAGE electrophoresis and transferred to nitrocellulose. Immunoblotting was performed with an anti-GST antibody (1:1000 dilution; Pharmacia). One of the clones that produced fusion proteins of the expected molecular weight was further characterized by nucleotide sequencing using vector-specific primers (Fig. 6, *clone #1* from *Pt1*).

Cloning and Sequencing of HERV-K RT-PCR Amplification Products. RT-PCR products resolved on agarose gels were purified using the QIAquick gel purification kit (Qiagen), subcloned into pCR-II vectors (Invitrogen, Carlsbad, CA), and sequenced using vector-specific reverse and forward primers plus internal HERV-K *env*-specific primers. Sequence analysis and alignment were carried out with DNAsis for Windows (Hitachi Software, San Francisco, CA) and the GenBank database.
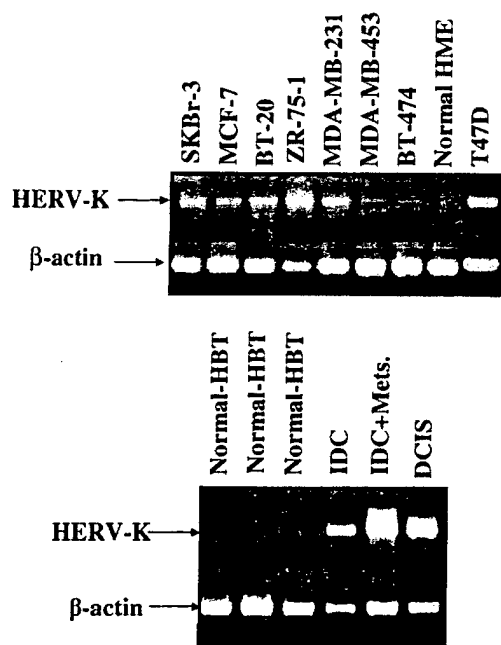
Fig. 2 Expression of HERV-K *env* RNA in breast cell lines and tissues. Ethidium bromide stained 1% agarose gel electrophoresis of RT-PCR products. Reverse transcribed RNA from breast cell lines and tissues was amplified by PCR (RT-PCR) using primers specific to the HERV-K envelope region and β-actin. *A*, RT-PCR amplification of RNA from SKBr-3, MCF-7, BT-20, ZR-75-1, MDA-MB-231, MDA-MB-453, BT-474, normal mammary epithelial cells (*HME*), and T47D. *B*, RT-PCR amplification of RNA from normal human breast tissues HBT (obtained from reduction mammoplasty) and breast cancer tissues including invasive ductal carcinoma (*IDC*, same as *Pt1* in Fig. 6), IDC with metastasis to lymph nodes (*IDC+Mets*, same as *Pt3* in Fig. 6), and DCIS (same as *Pt2* in Fig. 6).

Results

Detection of the *env* Region of HERV Transcripts in Breast Cancer Cell Lines and Tissues by RT-PCR. To investigate the expression of HERV elements in breast cancer cell lines, RT-PCR was performed using primers specific for *env* genes of ERV3, HERV-E4-1, and HERV-K. The HERV primer pairs for RT-PCR allowed detection of transcripts encoding the putative surface portion of each HERV env protein (Fig. 1). Expression of ERV3 and HERV-E4-1 RNA was not detected in breast tumor samples, although amplification of these products from placenta served as a positive control for PCR validation (data not shown). In contrast, HERV-K transcripts were detected in SKBr-3, MCF-7, BT20, ZR-75-1, MDA-MB-231 and T47D, but not in MDA-MB-453, BT-474, and normal HME cells (Fig. 2*A*). A single fragment of ~1100 bp, consistent with the size of a type 1 HERV-K *env* region, was detected. Because the primers were identical with several known type 1 HERV-K family members but contained 1–4 bp mismatches with known type 2 HERV-K env sequences, amplification of a single fragment was not unexpected. Expression of type 1 *env* transcripts in T47D cells has been reported previously (10).

These studies were next extended to surgical samples of breast carcinoma and nonmalignant breast tissues. Again,

Table 1 Detection of HERV-K *env* gene expression in human tissue samples by RT-PCR

Sample description	No. of samples	HERV-K positive	
		No.	(%)
Breast carcinoma	55	25	(45)
Normal breast tissue from breast carcinoma samples	40	7	(18)
Normal breast tissue from patients without carcinoma[a]	35	0	(0)
Placenta	12	10	(83)

[a] Samples were from reduction mammoplasty (*n* = 31) and biopsies of fibrocystic lesions (*n* = 4).

ERV-3 and HERV 4-1 transcripts were not detected in 43 breast tissue samples (20 tumor samples and 23 nonmalignant samples). In contrast, amplification products derived from the HERV-K *env* region were frequently detected (Fig. 2*B* and Table 1). To date, 130 breast tissue samples have been analyzed for expression of HERV-K transcripts by RT-PCR using the HERV *env* primers (Table 1). HERV-K *env* RNA was detected in 45% of breast cancer samples, in 18% of breast tissues derived from breast cancer patients but judged to be uninvolved by gross pathological dissection, as well as in 83% of placentas analyzed. In contrast, breast tissues derived from individuals not having breast cancer had no detectable HERV-K *env* expression (*n* = 35).

HERV *env* Expression in Breast Cancer Cell Lines and Tissues Evaluated by Northern Blot Analysis. Northern blot analysis was used to further assess expression of HERV-K transcripts (Fig. 3). Using the *env* region as a probe (99% homologous to HERV-K102), positive radioactive species corresponding to the full-length proviral transcript (~8.3 kb) and the putative spliced *env* mRNA transcript (~3.0 kb) were detected in human breast cancer tissues but not in normal breast tissue (see Fig. 3). As a positive control, the full-length and *env* transcripts were also detected in the teratocarcinoma cell lines Tera I and Tera II (data not shown).

Expression of *env* Localized by RNA ISH. To demonstrate that HERV-K RNA was transcribed specifically in breast tumor cells, we performed *in situ* hybridization. Serial tissue sections were prepared and hybridized with an HERV-K *env* probe. HERV-K RNA was detected by ISH in 7 of 10 RT-PCR-positive breast tumor samples. This difference in the percentage of positive samples may reflect the lower sensitivity of ISH as compared with RT-PCR or may reflect differences in the specific area of each tissue sample used for analysis in the two independent assay types. In the positive tumor samples, the hybridization signal was detected specifically in the tumor cells and not in the surrounding normal cells. More importantly, when so-called "uninvolved" areas of breast cancer patient tissues were examined (*i.e.*, normal breast tissue from breast carcinoma samples), it became apparent that the positive signal detected in some of these samples by RT-PCR (Table 1) was likely attributable to the presence of previously undetected malignant cells (data not shown). Examples of ISH results are presented in Fig. 4, where a strong positive signal is detected in tumor epithelial cells of a tissue sample containing ductal car-
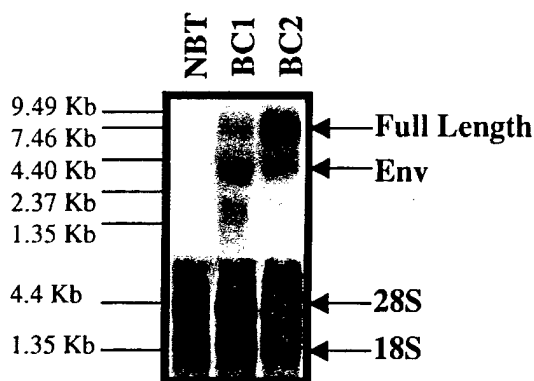
Fig. 3 Northern blot analysis of HERV-K *env* RNA expression in human breast tissues. Human tissues include normal human breast tissue (*NBT, Lane 1*) and two invasive ductal carcinoma samples (*BC1* and *BC2, Lanes 2* and *3*, respectively). Two transcripts are identified on the right. Top band, full proviral transcript. The bottom transcript presumably represents the spliced *env* mRNA. To verify the presence of RNA in all lanes, the blot was stained with methylene blue solution (*bottom panel*).

Fig. 4 Detection of HERV-K transcripts in breast specimens by *in situ* hybridization. Characterization of HERV-K transcriptional activity in serial breast tissue sections by *in situ* hybridization using a digoxigenin-labeled *env* RNA probe. *A* and *B*, breast ductal carcinoma *in situ*. *C* and *D*, uninvolved breast tissues. Sections depicted in *A* and *C* were hybridized with HERV-K *env* RNA antisense (*AS*) probes, whereas those depicted in *B* and *D* were hybridized with HERV-K RNA sense (*S*) probes. Sections were visualized by light microscopy.

cinoma *in situ* (Fig. 4*A*), but no signal is detected in the matched, uninvolved epithelial cells of the same tissue sample (Fig. 4*C*). Matched tissue sections analyzed with the control sense probe were negative (Fig. 4, *B* and *D*). In addition, if the tissue was first treated with RNase, there was no signal using the antisense probe (data not shown). As expected from RT-PCR and Northern blot results, breast tissue from six individuals not having breast cancer had no detectable HERV-K RNA by ISH analyses. Thus, HERV-K transcripts were only detected in tumor cells by this assay.

Production of HERV-K env Protein from cDNAs Derived from Breast Cancer Tissues. As a test of the coding potential of the above expressed *env* sequences, HERV-K *env* cDNA amplified from selected breast cancer samples was cloned into a prokaryotic GST-fusion protein expression vector. The predicted recombinant fusion proteins would contain M_r ~26,000 of GST plus M_r ~41,000 daltons of HERV-K env surface protein. Six independent clones derived from a single breast tumor specimen (Fig. 6, Pt #1) were analyzed. Of these, four clones produced a fusion protein of the expected molecular size (M_r ~67,000), one produced a fusion protein of lower than predicted size, and one produced protein of the size of GST alone (partial results in Fig. 5). Similar results were obtained using clones derived from four additional breast tumor samples. HERV-K *env* cDNAs derived from the same tumor sample (Pt 1) were cloned into a eukaryotic expression vector for assay by coupled *in vitro* transcription-translation with [^{35}S]methionine. Radiolabeled protein of the size expected (M_r ~40,000) for the targeted HERV-K env product was detected in two additional clones (data not shown; sequence analysis in Fig. 6, *Pt1, clones #2* and *#3*). These results suggest that the amplified HERV-K *env* regions contained no premature stop codons and provide indirect evidence that such HERV-K env proteins can be stably expressed.

Sequence Analysis of HERV-K Clones Derived from Breast Cancer Tissues. HERV-K *env* RT-PCR products derived from four individual breast cancer patient tissue samples were cloned, and eight independent isolates were completely sequenced (Fig. 6). All demonstrated >97% sequence homology to the previously reported HERV-K102 *env* (35), but none were identical to any published sequence. Sequence divergence from K102 was noted at 46 of 1035 nucleotides analyzed (4.4%). Of note, none of the isolated clones were identical to each other, even among clones derived from the same tumor sample, although specific nucleotides sequence divergence from K102 was in many cases shared among the eight clones. Specifically, nucleotide divergence was identical with at least one of
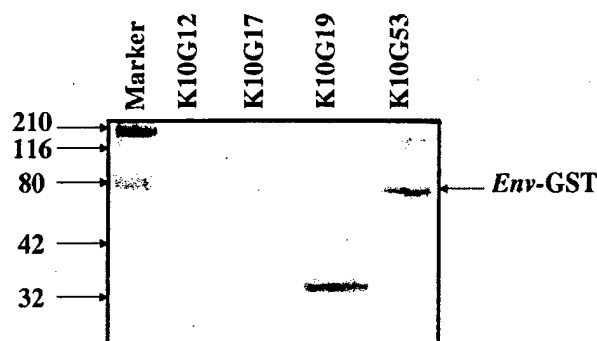
Fig. 5 Production of HERV-K env protein from a prokaryotic vector. Ten μg of HERV-GST fusion protein purified by glutathione Sepharose 4B chromatography were electrophoresed on a 12% SDS-PAGE gel and analyzed by Western blot. Shown are four of six independently isolated clones. Lanes *1*, *2*, and *4* are full-length fusion proteins produced by clones K10G12, K10G17, and K10G53, respectively. Clone K10G19 in *Lane 3* did not produce a full-length protein. *Lane M*, Kaleidoscope prestained standards (Bio-Rad; molecular weight in thousands).

the other seven isolates for 13 of 46 nucleotide positions (28%). The remaining 33 nucleotide changes (72%) were unique among the eight cDNA clones. A few cDNA sequences showed highest homology to published HERV-K type 1 genes other than K102 (*e.g., Pt1 clone #3* in Fig. 6 was most homologous to K101, GenBank accession # M14123), but in no case did the sequence homology exceed 99.5%.

Discussion

Endogenous retroviruses have several potential functional roles in their host. By analogy with mouse models of cancer, we hypothesized that proteins encoded by HERV *env* genes may act as tumor antigens. As a first test of this hypothesis, we used RT-PCR to examine expression of the *env* region of several candidate HERV genes in human breast cancer cell lines and surgical specimens. Whereas no breast tissue expression of ERV3 or HERV-E4-1 was found in our analyses, RT-PCR readily detected HERV-K transcripts in six of eight breast carcinoma cell lines and in 45% of the 55 analyzed breast tumor tissue samples. In contrast, a normal breast epithelial cell culture (HME) and multiple breast tissue samples from individuals not having breast cancer showed no detectable HERV-K *env* RNA. Northern blot analysis using an *env* probe derived from a breast tumor sample confirmed expression of HERV-K transcripts in breast cancer. Because a small percentage of samples judged to be nonmalignant by gross pathological inspection had detectable *env* RNA by the RT-PCR assay (18%), ISH was used to localize expression of HERV-K transcripts to individual cells by histological discrimination between tumor and adjacent uninvolved tissues. Such ISH studies indicated that small numbers of tumor cells present in some matched tissue samples previously identified as "uninvolved" based upon gross inspection could account for positive HERV-K *env* RT-PCR results for these samples (as in Table 1). Considered together, our RT-PCR and ISH results support the conclusion that expression of HERV-K transcripts is restricted to breast carcinoma and is undetectable in nonmalignant breast epithelial cells.

Expression of endogenous retroviral sequences has been implicated in a variety of human disease states (36). Of particular relevance to the current study, Yin *et al.* (37) previously described expression of HERV-K sequences in both normal and malignant breast tissue, using a classification system and assay targeting the *pol* genes of HERV-K subgroups HML 1–6. They reported that, on average, all HML groups were expressed at lower levels in breast tissues as compared with placenta, and furthermore, that HML *pol* genes were not more highly expressed in malignant as compared with nonmalignant breast tissues. Although the conclusions of Yin *et al.* (37) may seem in conflict with those from the current study, it should be noted that different retroviral gene products (*pol versus env*) and different HERV-K subgroups (HML *versus* K102) were targeted. It might be expected that gene-specific expression for individual HERV-K elements would correlate with overall transcriptional activity of HERV-K loci in any given cell type; however, this presumption is untested in the available literature and remains unexamined for samples used in the current study.

Whereas expression of various HERV mRNA species has been reported previously in different tumor tissues, HERV protein expression has been confirmed in only a minority of cases. Most HERV loci are thought to be defective, and certain HERV-K *env* sequences, including HERV-K10 and HERV-K107 (both type 1), are reported to contain a premature stop codon (TAA at nucleotide 6920) that should ablate env protein expression. The *env* cDNA clones sequenced in our study contained no stop codon at this position (codon was CAA instead of TAA for all eight clones), and no stop codons were observed over the entire *env* region analyzed in the presumed reading frame. Furthermore, our *in vitro* studies suggest that the HERV-K *env* cDNA clones from breast cancer tissue were capable of producing stable env protein of the predicted molecular size. Others have also reported expression of HERV-K env protein from cloned *env* cDNA after transfection into a mouse cell line (38). Thus, the potential for expression of HERV-K env proteins in human breast cancer is supported by independent data.

Sequencing of *env* cDNAs in the current study was performed in part to determine whether the transcripts were likely to have arisen from a single locus. Within the region of the HERV-K *env* gene sequence analyzed, the eight completely sequenced cDNAs amplified from breast cancer tissues showed >97% nucleotide homology to HERV-K102. Significant homology with HERV-K101, HERV-K10, and *env* sequences located on chromosomes 5 and 19 was also noted for individual clones. However, none of the eight sequenced clones were identical to each other or to any published *env* sequence. Although our sample size is small, it is interesting to note that many of the nucleotide changes were shared among clones isolated from either the same or an unrelated patient tumor specimen. Although it is possible that some of these nucleotide differences may have been generated during the amplification and cloning process, it is unlikely that this would account for all of the differences, particularly those specific changes observed in multiple clones. Also, inherited polymorphisms in HERV-K genes would not explain the cDNA sequence divergence among clones isolated from the same patient tumor sample. Random mutagenesis during the malignant transformation of breast tu-

tumor cDNA clones	HERV-K102 genomic sequence, nt number																																															% difference from HERV-K102	
	6511	6530	6547	6549	6562	6567	6624	6632	6650	6654	6655	6657	6711	6787	6794	6874	6883	6929	6934	6994	7000	7031	7052	7077	7105	7112	7114	7119	7139	7244	7254	7262	7285	7286	7298	7300	7334	7352	7388	7392	7431	7483	7494	7495	7497	7513	nt	aa
Consensus	G	A	T	T	A	T	T	C	C	G	G	G	C	A	T	A	T	G	T	T	G	G	T	T	A	A	A	C	G	T	T	A	A	G	A	T	A	G	C	G	T	A	C	A	C	G		
Pt1 #1	C																	G		C																					T						0.4	0.6
Pt1 #2		G	C	A	C	C															G																					C					0.7	1.4
Pt1 #3		G	C	A	C	C				T			C	T		C		A	C	C	A	A	C			G			A	C	G	C		G			C		G		T	A					2.4	4.6
Pt2 #1				C		C																									G								C	C							0.5	1.2
Pt2 #2		G	C		C	C					T		T			A	C	A							G		A	C		G					T	T	C		G	T	G						1.8	3.5
Pt3 #1				C				A				G								T							G	G								C						C					0.6	0.6
Pt4 #1				C								G		G	G								G					C																			0.6	1.2
Pt4 #2		G	C	A	C	C															G																				C						0.7	1.4

Fig. 6 Analysis of nucleotide sequences of cloned RT-PCR amplifications of breast cancer patients. Sequence alignments were performed using DNAsis (Hitachi Software). HERV-K env clones derived by RT-PCR of patient (*Pt*) tumor tissue RNA were sequenced as described in the text. Consensus cDNA sequences were aligned with the HERV-K102 genomic sequence, GenBank accession no. AF164610 (35), using genomic sequence numbering spanning nucleotides 6494–7528. Only cDNA nucleotide differences from the HERV-K102 genomic sequence are shown, with each presumed amino acid (*aa*) difference denoted by a *shaded box*. No nucleotide deletions or insertions were observed in the analyzed cDNA clones with respect to the HERV-K102 genomic sequence. Pathological classifications of tumor samples used for cDNA cloning were as follows: *Pt1*, invasive ductal carcinoma; *Pt2*, ductal carcinoma *in situ*; *Pt3*, invasive ductal carcinoma with metastasis to lymph node; *Pt4*, invasive ductal carcinoma.

mor precursors would not seem to explain the identical nucleotide changes observed among clones derived from four individual patients. Because genomic sequences are not yet available for all of the estimated 30 or more copies of HERV-K in the human genome, it is difficult to estimate the potential impact of allelic variation on the current study. Considering the minimal estimated complexity of HERV proviral sequences within the human genome (39), clarification of the precise origins of the expressed *env* sequences studied here awaits more complete sequence data provided by the Human Genome Project.

Collectively, our results are in agreement with the hypothesis that multiple, related HERV-K gene loci may be transcriptionally activated in breast tumors. Such activation might occur because of similarities in HERV-K long terminal repeats, which might be activated in response to a transcriptional factor found specifically in the altered transcriptional milieu of malignant breast epithelial cells (40, 41). Alternatively, activation may be attributable to hypomethylation of genomic DNA. HERV-K sequences were reported to be hypomethylated in urothelial carcinomas (42), suggesting that a similar mechanism may function in breast carcinoma. Further investigations of the mechanisms regulating expression of the multiple individual HERV loci are needed to define those most likely to be active in human malignancy.

In summary, HERV-K *env* RNA was expressed in many of the breast cancer tissues and cell lines analyzed, with no expression detected in normal breast tissues, suggesting that *env* expression might be a tumor marker. These transcripts are likely to originate from multiple HERV-K loci, even within the same tumor. Similar analyses with expanded tumor samples will be needed to confirm these findings, to estimate the number of individual HERV-K loci transcribed in tumors, and to establish the potential prognostic or diagnostic value of *env* RNA and/or protein expression. Investigation of the mechanism resulting in HERV-K transcriptional activation in tumor cells has the potential to elucidate fundamental cell processes involved in malignant transformation.

Acknowledgments

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